Original Research

Limit of Detection Test on Salmonella spp testing on Processed Food Products Egg Pindang According to ISO 16140-3: 2021

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Abstract—*The development of pathogenic bacteria detection methods in food products has led to the emergence of faster, shorter, and more efficient techniques. However, when choosing a reference method for testing, it is crucial to ensure its reliability. The purpose of this study was to determine the limit of detection (LOD) for Salmonella testing in processed chicken egg food products (Pindang Egg). The research aims to provide valuable information and serve as a reference for similar studies. The method used in this study follows ISO 6579-1:2017, while the detection limit is determined according to ISO 16140-3:2021. The results from observations on pre-enrichment and enrichment media showed that all inoculated samples exhibited a color change to cloudy, indicating bacterial growth in the media. Isolation on selective media and conformational tests confirmed 100% positivity for all samples in contamination variations of 11, 3.67, and 1.22. However, for contamination variation of 0.41, only 75% of the analyzed test data detected the presence of Salmonella, with 1 out of 4 replications not detecting it (approximately 20% failure rate). In conclusion, the lowest LOD value that can be reliably detected is 100% in the contamination variation of 1.22, while for the 0.41 variation, the detection rate only reaches 75% of the analyzed test data.*

Keywords: *bacteria, eggs, pathogen, salmonella*

Abstrak—Pengembangan metode deteksi bakteri patogen dalam produk pangan telah menghasilkan teknik yang lebih cepat, lebih singkat, dan lebih efisien. Namun, saat memilih metode referensi untuk pengujian, sangat penting untuk memastikan kehandalannya. Tujuan dari penelitian ini adalah untuk menentukan batas deteksi (LOD) untuk pengujian Salmonella dalam produk pangan telur ayam olahan (Pindang Egg). Penelitian ini bertujuan untuk memberikan informasi berharga dan menjadi referensi untuk penelitian serupa. Metode yang digunakan dalam penelitian ini mengikuti ISO 6579-1:2017, sementara batas deteksinya ditentukan sesuai dengan ISO 16140-3:2021. Hasil pengamatan pada media pra-pemupukan dan pemupukan menunjukkan bahwa semua sampel yang diinokulasi menunjukkan perubahan warna menjadi keruh, menandakan pertumbuhan bakteri dalam media. Isolasi pada media selektif dan uji konformasi mengkonfirmasi positivitas 100% untuk semua sampel dalam variasi kontaminasi sebesar 11, 3,67, dan 1,22. Namun, untuk variasi kontaminasi sebesar 0,41, hanya 75% data uji yang dianalisis mendeteksi keberadaan Salmonella, dengan 1 dari 4 replikasi tidak mendeteksinya (sekitar 20% tingkat kegagalan). Sebagai kesimpulan, nilai LOD terendah yang dapat dideteksi dengan handal adalah 100% dalam variasi kontaminasi sebesar 1,22, sementara untuk variasi 0,41, tingkat deteksinya hanya mencapai 75% dari data uji yang dianalisis.

Kata kunci: bakteri, telur, patogen, salmonella

INTRODUCTION

The emergence of various methods for detecting pathogenic bacteria in food products has led to faster, shorter, and more efficient techniques. However, when choosing a reference method for testing, it is crucial to ensure its reliability. One way to test the reliability of a method is through verification and validation. The determination of the detection limit is one of the parameters in the validation process.

Several studies on the validation method for identifying Salmonella spp. have been carried out (Sturza et al., 2021; Malorny et al., 2003; Löfström et al., 2012; Löfström et al., 2009; Coelho et al., 2021; Carlin et al., 2020). The quality of the testing method can be assessed based on the sensitivity of the Limit of Detection (LOD) that can be detected. While detection limit tests on microbiological testing have been conducted for various tests such as E. coli (Sophian, 2022) and Salmonella (Hantash et al., 2020), specific tests for Salmonella in processed egg products have not been found. So far, Salmonella detection tests have been conducted using various molecular testing techniques in health supplement products (Sophian

et al., 2020), traditional medicine (Sophian et al., 2021), and food (Lins 2018; Taskila et al., 2012; Hantash et al., 2020; Sophian & Muindar, 2021; Sophian et al., 2022; Sophian et al., 2023). Additionally, rapid test kits have also been used for Salmonella detection (Hantash et al., 2020; Zhao et al, 2021; Sophian & Muindar, 2021). However, information on the detection limit is essential to serve as a reference in determining the sensitivity of the test method used.

In this study, the method used refers to ISO 6579-1:2017 (British Standard, 2020) and ISO 16140-3:2021 (British Standard, 2021) utilizing pre-enrichment techniques on BPW media, enrichment on MKTNN and RVS media, selective media isolation, and confirmation tests using API 20E. Based on the background mentioned above, this research aims to determine the detection limit for Salmonella testing in processed food products, specifically chicken eggs (Pindang Egg). The research is expected to serve as a valuable source of information and reference for similar studies. Determining the LOD value is crucial in producing a robust test method that can be used to assess the quality of a product effectively.

MATERIALs AND METHODS

Materials

Pindang egg samples were 17 packages weighing 25 grams each, Media Buffered Peptone Water (BPW), Rappaport Vasiliadis Medium + Soya (RVS), Muller Kaufmann Tetrathionate Novobiocin Broth (MKTTn), Xylose Lysine Deoxycholate (XLD), Tryptic Soy Agar (TSA), API 20E Kit.

Standard microbes

The standard microbial *Salmonella Typhimurium* ATCC 14028 (WDCM 00031) was grown on TSA plate media and incubated at 36±10C for ±24 hours. Furthermore, the standard microbes were made into a suspension with a turbidity of 1 Mc Farland in 0.85% NaCl (equivalent to 2.0 x 108 CFU/mL).

Work procedures

The working procedure and test protocol used in this study refer to ISO6579-1-2017 and the detection limit refers to ISO 16140-3-2021.

Homogenization and Pre-Enrichment

A total of 25 g of sample was weighed into a suitable sterile container, then 225 mL of BPW was added, homogenized and then incubated at 34-38°C for 18±2 hours.

Enrichment

Each pre-enrichment culture was pipette 0.1 mL into 10 mL RVS then incubated at 41.5±1 °C for 24 ± 3 hours and 1 mL into 10 mL MKTTn then incubated at 37±1 °C for 24± 3 hours.

Isolation on Selective Media

Cultures from each enrichment were inoculated as much as 1 loop on the surface of the XLD medium and then incubated at 34-38 °C for 24 ± 3 hours, with the plates inverted.

Confirmation Test

Select one to five suspected colonies from the XLD media dish for confirmation. Suspected colonies were inoculated onto TSA media. The plates were incubated at 34–38 °C for 21 ± 3 hours. The confirmation test is carried out using the API 20E kit according to the kit manual.

Limit of Detection

LOD was evaluated by adding *Salmonella Typhimurium* suspension to samples with various concentration levels of 11 colonies/mL: 3.47 colonies/mL; 1.22 colonies/mL; 0.41 colony/mL (one-third of the previous concentration) was assumed to be the LOD value. Repeated analysis was performed on each of these concentrations. The lowest concentration of contamination in the sample that can be detected by the ISO 6579:2017 method is determined as the LOD estimate by calculating the replications that give positive results. Of the four concentrations, 3 high, medium and low concentrations were determined. The variations taken are 11; 3.47; and the lowest is 1.22. Based on ISO 16140-3:2021 it is determined that the estimated LOD 1:4:4 is < 1 x the lowest concentration in this test 1.22 so the estimated LOD for Salmonella detection tests in egg products is < 1.22 colonies/g..

RESULTS

Test Results on Pre-Enrichment Media

Observations on pre-enrichment media showed that all samples inoculated on BPW pre-enrichment media showed a colour change from brownish to cloudy. The results of the observations are more complete as presented in table 1 below.

Table 1

Interpretation of BPW Pre-enrichment Media

Test	Variation of contamination (cfu/ 25 g sample)				Negative Sample
	11	3,67	1,22	0,41	
1	cloudy	cloudy	cloudy	cloudy	cloudy
2	cloudy	cloudy	cloudy	cloudy	[redacted]
3	cloudy	cloudy	cloudy	cloudy	
4	cloudy	cloudy	cloudy	cloudy	

Results on RVS and MKTTN Enrichment Media

Observations on enrichment media showed that all samples inoculated on RVS pre-enrichment medium showed a colour change from turquoise to turbid. This change indicates a growth in the enrichment medium. The results of the observations are more complete as presented in table 2 below.

Table 2

Interpretation of RVS Enrichment Media

Test	Variation of contamination (cfu/ 25 g sample)				Negative Sample
	11	3,67	1,22	0,41	
1	+	+	+	+	-
2	+	+	+	+	[redacted]
3	+	+	+	+	
4	+	+	+	-	

Description: + (The media turned cloudy), - (Media turns blue)

The results of observations on enrichment media showed that all samples inoculated on MKTT pre-enrichment media showed a colour change from greenish-white to cloudy. This change indicates a growth in the enrichment medium. The results of the observations are more complete as presented in table 3 below.

Table 3
Interpretation of MKTTN Enrichment Media

Test	Variation of contamination (cfu/ 25 g sample)				Negative Sample
	11	3,67	1,22	0,41	
1	+	+	+	+	-
2	+	+	+	+	
3	+	+	+	+	
4	+	+	+	-	

Description: + (The media turned cloudy), - (Media turns clear)

The results of observations on selective media showed that all samples of contamination variations 11, 3.67, and 1.22 which were inoculated on XLD pre-enrichment media showed colony growth with red characteristics with or without black spots. This change indicated that there was growth in the isolation medium with characteristics that showed positive Salmonella results, while for contamination variations of 0.41, from 4 replications 1 of them did not show growth or around 20%. The results of the observations are more complete as presented in table 4 below.

Tabel 4
Intrepretasi Media Selektif XLD

Test	Variation of contamination (cfu/ 25 g sample)				Negative Sample
	11	3,67	1,22	0,41	
1	+	+	+	+	-
2	+	+	+	+	
3	+	+	+	+	
4	+	+	+	-	

Description: + (Red colony with or without a black spot in the center), - (No colony growth)

Results on conformational tests using API 20 E showed that all samples of contamination variations 11, 3.67, and 1.22 were confirmed to be positive and detected 100%, while for contamination variations of 0.41, out of 4 replicates 1 of them was not detected or around 20%. The results of the observations are more complete as presented in table 5 below.

Table 5
Confirmation Test Using API 20 E

Test	Variation of contamination. (cfu/ 25 g sample)				Negative Sample
	11	3,67	1,22	0,41	
1	+	+	+	+	-
2	+	+	+	+	
3	+	+	+	+	
4	+	+	+	-	

Description: + (Identified *Salmonella* spp.), - (Not identified *Salmonella* spp.)

DISCUSSION

Selective media isolation in this study used XLD, this is because the media can inhibit gram-positive bacteria because it contains sodium deoxycholate, and contains thiosulfate as an

H2S indicator which is seen in colonies growing in XLD media. On the XLD medium, Salmonella colonies were translucent and round with a black spot in the middle (Maddocks et al., 2002; Nye wt al., 2002; Sophian et al., 2021). The colour change that occurs is due to the fermentation process of glucose by Salmonella into organic acids such as lactic, acetic and formate acids, resulting in a decrease (Sophian et al., 2021). Salmonella typhimurium uses xylose carbohydrates in XLD media to carry out fermentation activities and conditions the pH of the media to become acidic and then will decarboxylate lysine so that it increases the pH to become alkaline. Meanwhile, Salmonella typhimurium uses decarboxylate enzymes to produce amines or diamines and carbon dioxide by breaking down amino acid groups (Sophian et al., 2020; Sophian et al., 2022; Sophian et al., 2023)]. XLD is a non-autoclave selective medium, this is because the components of its constituent media will be damaged when the heating process is carried out at a temperature of more than 10 ^{o}C.

Xylose-Lysine-Desoxycholate agar was originally formulated by Taylor1 for the isolation and identification of shigella from faecal specimens. It has since been found to be a satisfactory medium for the isolation and identification of suspected salmonellae and shigellae. It relies on xylose fermentation, lysine decarboxylation and hydrogen sulfide production for the major differentiation of shigellae and salmonellae from non-pathogenic bacteria. On XLD media, Salmonella colonies were red with black spots, this was because Salmonella could ferment xylose and decarboxylate lysine so it changed the pH to alkaline causing the media to be red. presence of Salmonella and Edwardsella spp. distinguished from shigellae by the formation of hydrogen sulfide. The high acid level produced by the fermentation of lactose and sucrose prevents lysine-positive coliforms from returning the pH to an alkaline value, and non-pathogenic hydrogen sulfide producers do not decarboxylate lysine. The acid level also prevents discolouration by these microorganisms until after 18-24 hours of testing. Sodium desoxycholate was included as an inhibitor in the medium. The concentration used allows the inhibition of coliforms without compromising the ability to support shigella and salmonellae.

LOD was evaluated by adding *Salmonella Typhimurium* suspension to samples with various concentration levels of 11 colonies/mL: 3.47 colonies/mL; 1.22 colonies/mL; 0.44 colony/mL (one-third of the previous concentration) was assumed to be the LOD value. Repeated analysis was performed on each of these concentrations. The lowest concentration of contamination in the sample that can be detected by the ISO 6579:2017 method is designated as LOD

CONCLUSION

Based on the results of these studies, it can be concluded that the lowest LOD value that can be detected is 100% in the contamination variation of 1.22, while for the 0.41 variation, the detection rate only reaches 75% of the analyzed test data.

The recommendation from this study is that it is recommended to validate/verify the method following the LOD test data that has been carried out so that more comprehensive information is obtained about the test method used for testing the detection of Salmonella spp in egg-processed food products (Pindang Egg). It is also hoped that this LOD data can be used as a reference for information in the detection limit test.

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